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Reassessing the evolutionary history of ass-like equids: Insights from patterns of genetic variation in contemporary extant populations

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ABSTRACT

All extant equid species are grouped in a single genus – *Equus*. Among those, ass-like equids have remained particularly unstudied and their phylogenetic relations were poorly understood, most probably because they inhabit extreme environments in remote geographic areas. To gain further insights into the evolutionary history of ass-like equids, we have used a non-invasive sampling approach to collect representative fecal samples of extant African and Asiatic ass-like equid populations across their distribution range and mitochondrial DNA (mtDNA) sequencing analyses to examine intraspecific genetic diversity and population structure, and to reconstruct phylogenetic relations among wild ass species/subspecies. Sequence analyses of 410 base pairs of the fast evolving mtDNA control region identified the Asiatic wild ass population of Kalamaili (China) as the one displaying the highest diversity among all wild ass populations. Phylogenetic analyses of complete cytochrome *b* sequences revealed that African and Asiatic wild asses shared a common ancestor approximately 2.3 Mya and that diversification in both groups occurred much latter, probably driven by climatic events during the Pleistocene. Inferred genetic relationships among Asiatic wild ass species do not support *E. kiang* monophyly, highlighting the need of more extensive studies in order to clarify the taxonomic status of species/subspecies belonging to this branch of the *Equus* phylogeny. These results highlight the importance of re-assessing the evolutionary history of ass-like equid species, and urge to extend studies at the population level to efficiently design conservation and management actions for these threatened species.

1. Introduction

Equids (i.e. Equidae family) including the extant horses, donkeys or asses, and zebras, and many other extinct horse-like species have been the subject of numerous studies over the past two centuries. The rich fossil record from the extinct horse-like species has vividly demonstrated a classic example of long-term evolutionary changes and has also been a theme of debate among academics since the end of the nineteenth century (MacFadden, 2005). According to currently accepted IUCN taxonomy (Moehlman, 2002), modern equids are represented by the eight extant species of the *Equus* genus: domestic horse (*E. caballus*), Przewalski's horse (*E. przewalskii*), kiang (*E. kiang*), Asiatic wild ass (*E. hemionus*), African wild ass (*E. africanus*), mountain zebra (*E. zebra*), plains zebra (*E. quagga*) and Grevy's zebra (*E. grevyi*). Under this taxonomy, the domestic donkey (*E. africanus asinus*) is considered a subspecies of African wild ass. Molecular studies in the past three decades (George and Ryder, 1986; Kruger et al., 2005; Oakenfull and Clegg, 1998; Oakenfull et al., 2000, 2006, 2009; Steiner et al., 2012; Steiner and Ryder, 2011;

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Vilstrup et al., 2013) have focused on resolving the complex phylogeny of all extant equids and understanding their evolutionary history, however this has proved to be a challenging task. Besides the confounding effect of incomplete lineage sorting – as a result of rapid and recent divergence – and the probable past and/or present introgression among sympatric species, available phylogenetic studies on the *Equus* genus have often relied on a limited number of samples obtained from zoo collections or captive individuals with few available information about their exact geographical origin (Oakenfull et al., 2000; Steiner et al., 2012; Steiner and Ryder, 2011). The ass-like branch of the equid phylogeny including Asiatic (*E. hemionus* and *E. kiang*) and African (*E. africanus*) wild asses remains a particularly understudied group of species.

Historically, it is accepted that the Asiatic wild ass ranged from the Arabian peninsula to the Manchuria in a continuous distribution (Grubb, 2005). However, over the time, climatic events and increasing anthropogenic impact on their habitats have fragmented their distribution. As a result these species are now scattered in small and isolated populations that are located in arid or high altitude areas in Iran, Turkmenistan, Mongolia, China, and India.

Limited scope studies based on morphology, coat color, geographic location and chromosomal number have been used to justify the split of the Asiatic wild ass into two distinct species – *E. hemionus* and *E. kiang* (Bennett, 1980; Groves, 1974; Ryder and Chemnick, 1990). Nonetheless, molecular data has provided no support for this distinction, with both mitochondrial and genomic data showing *E. kiang* individuals grouping together in a monophyletic clade inside the wider *E. hemionus* variation (McCue et al., 2012; Oakenfull et al., 2000; Vilstrup et al., 2013). Additionally, according to geographical range, three subspecies of *E. kiang* have been proposed (Groves and Mazák, 1967): *E. k. kiang*, *E. k. holdereri*, and *E. k. polyodon*, corresponding to Western kiang, Eastern kiang, and Southern kiang, respectively. The validity for these subspecies designations has also been questioned (Schaller, 1998; Shah, 2002).

It is of particular interest the question regarding the relative position of the African wild ass in the *Equus* phylogeny. Phylogenetic trees based on mtDNA and nuclear loci (Steiner et al., 2012) have either placed this species among zebras (mtDNA) or as the earliest diverging taxon of a monophyletic group that comprises all ass-like equids (nuclear loci). African wild asses are phenotypically variable, with two recognized extant subspecies – the Nubian wild ass (*E. a. africanus*) and the Somali wild ass (*E. a. somaliensis*) (Marshall, 2007; Moehlman et al., 2008b) – occupying distinct geographic areas. Additional genetic data from extant African wild ass populations is required to further assess this taxonomic designation, however political instability in the remote territories occupied by putative extant Nubian wild ass populations has made this task unachievable.

At a time when four out of the seven extant wild equid species are recorded as threatened in the IUCN Red List and conservation resources are limited, it is critical to have a clear understanding of genetic background of equid species and populations in order to appropriately prioritize conservation actions. The use of conservation units has helped in overcoming the taxonomic riddle that many times precludes the establishment of conservation programs at the species/subspecies levels (Crandall et al., 2000). The concept of evolutionary significant unit (ESU) has been debated over time, with different authors emphasizing the importance of adaptive distinctiveness (Crandall et al., 2000) over the concept of geographic discrete populations or the existence of reciprocal monophyly among proposed conservation units (Moritz, 1994). Modern approaches to the definition of ESUs have reinforced the need to combine data from neutral and adaptive markers in order to achieve optimal management decisions (Funk et al., 2012; Palsbøll et al., 2007).

Despite the advances in non-invasive methodologies (Beja-Pereira et al., 2009) and the growing impact of genomic approaches in conservation genetics (Allendorf et al., 2010), quantifying divergence at candidate adaptive loci is still a difficult task when using low quality/quantity DNA samples. In such cases, relying on phylogeographic data to define units of conservation might be a necessary first step in an ongoing and complex process.

To further clarify questions regarding the evolutionary history, taxonomy and conservation of the ass-like equid group we conducted a comprehensive study integrating phylogeographic and phylogenetic methodologies, using noninvasive sampling to obtain mtDNA sequences from extant natural populations of African and Asiatic wild asses. We have analyzed samples from 10 populations (see Table 1) representing the African and Asiatic taxa across its entire distribution range (Fig. 1A).

Given the overall decrease in wild equid populations (IUCN, 2014) and their level of endangerment it is of crucial importance to have an accurate knowledge about current levels of diversity among extant populations, as well as clarifying the taxonomic status of species. Such actions, along with more reliable information about the ecology of populations, will serve as an important step for prioritizing scientifically based conservation actions, such as the definition of ESUs (Moritz, 1994). This is critical for the prevention of further losses in the evolutionary potential of wild equid species.

2. Materials and methods

2.1. Sample collection

We collected fecal samples from three African and seven Asiatic wild ass populations, representing three species: two from Asia (*E. hemionus* and *E. kiang*), and one from Africa (*E. africanus*) (Table 1, Fig. 1). Fecal samples were collected in the field and placed in individual bags. The geographical location (GPS) of each sample was recorded as well as any other relevant information. Samples were dried naturally and stored at room temperature until further processing.

2.2. DNA extraction and PCR amplification

To minimize potential contamination issues inherent to non-invasive samples, DNA extraction was carried on a laminar flux chamber, physically separated from the PCR room. The samples were processed in batches with a maximum of 16 samples per set. All material used during the extraction process was sterilized between sample processing. In each batch of sample DNA extraction, a negative control containing all reagents but not the sample was included to detect contaminations. DNA extraction was carried using an adapted protocol from JetQuick™ Tissue DNA Spin Kit (Genomed, GmbH). Briefly, the modifications to the standard procedure consist of a pre-treatment with extraction buffer and proteinase K, followed by the use of an inhibitEX® tablet (QIAGEN, GmbH, Hilden). Primers Donk_F (CCC AAG GAC TAT CAA GGA AG) and CR_2R (GGA ATG GCC CTG AAG AAA G) were used to amplify a 440 bp fragment of the hypervariable region 1 (HVRI) of the mtDNA control region. Complete cytochrome *b* (Cyt *b*) sequences (1140 bp) were obtained by amplifying two overlapping fragments with the following primers: EaCB32F (AAG AAC ACT AAT GAC AAA CAT CC), EaCB687R (GGT GGA ATG GGA TTT TGT C), EaCB625F (TCC ATC TAC TAT TCC TCC ACG) and EaCB997R (CAA GAC CAG GGT AAT GTG TG). PCRs were performed in a 20 µl volume containing 1 × PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.6 µM each primer, 0.3 U of PlatinumTaq DNA Polymerase (Invitrogen), and variable amounts of genomic DNA. The PCR mixture underwent 10 min at 95 °C, 40 cycles of 45 s. at 95 °C, 60 s at 55 °C, 45 s at 72 °C, and a final 20 min at 72 °C on a GeneAmp

Table 1
Summary statistics of sampled wild ass populations. The sample information includes country of origin, population and number of samples. Nucleotide diversity (π), haplotype diversity (h), the population parameter θ_W (Watterson, 1975) and Tajima's D (Tajima, 1989) were calculated using mtDNA HVRI sequences.

Species/subspecies	Country	Population	n	Number of haplotypes	Number of segregating sites	h	π	θ_W	D
Equus africanus somaliensis	Ethiopia	Afdera	41	3	8	0.261 ± 0.085	0.0032 ± 0.0013	0.0045 ± 0.0020	−0.823
		Hillu	6	2	6	0.333 ± 0.215	0.0049 ± 0.0032	0.0064 ± 0.0038	−1.367
	Eritrea	Denkelia	38	4	9	0.711 ± 0.045	0.0082 ± 0.0010	0.0052 ± 0.0023	1.665
Equus hemionus onager	Iran	Touran	14	2	2	0.440 ± 0.112	0.0021 ± 0.0006	0.0015 ± 0.0012	1.079
		Bahram Gour	28	3	19	0.140 ± 0.087	0.0035 ± 0.0030	0.0119 ± 0.0045	−2.488*
Equus hemionus hemionus	China	Kalamaili (Xinjiang)	54	6	24	0.767 ± 0.038	0.0173 ± 0.0021	0.0129 ± 0.0043	1.104
Equus kiang	China (Tibet)	Nyalam	9	3	5	0.667 ± 0.132	0.0052 ± 0.0013	0.0045 ± 0.0026	0.625
		Tingri	8	1	0	0.000 ± 0.000	0.0000 ± 0.0000	0.0000 ± 0.0000	nc
		Gyirong	5	2	3	0.4 ± 0.237	0.0029 ± 0.0017	0.0035 ± 0.0025	−1.048
		Ngamring	4	2	1	0.5 ± 0.265	0.0012 ± 0.0007	0.0013 ± 0.0013	−0.612

* $P < 0.001$.

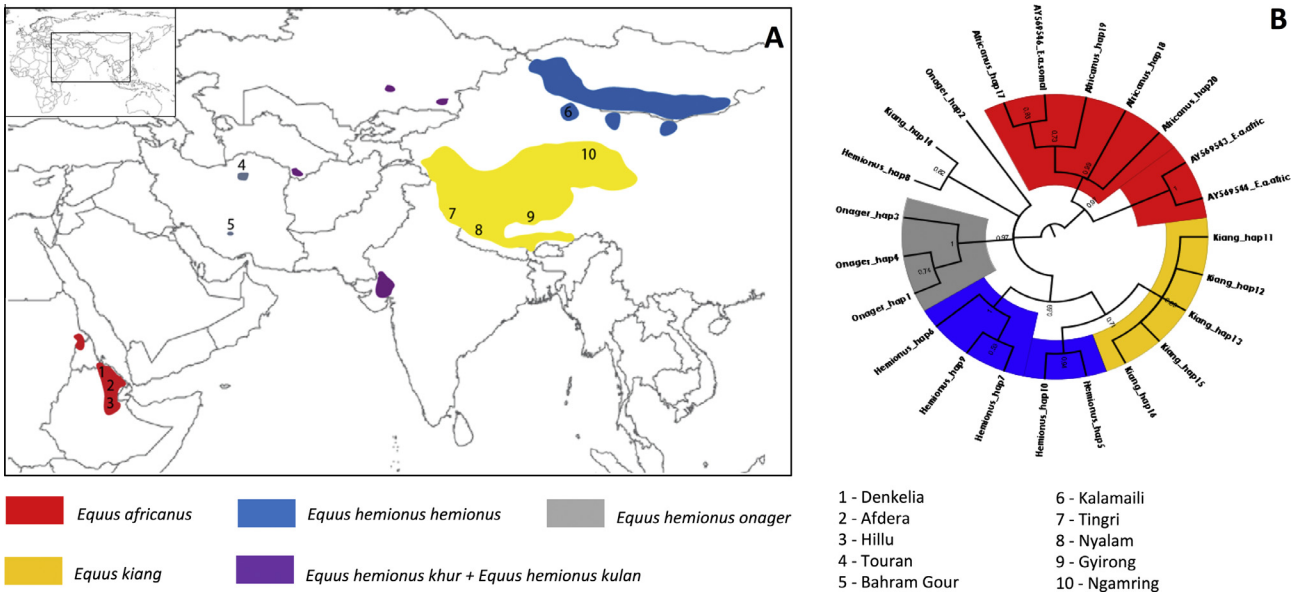

Fig. 1. Current distribution ranges of wild-ass species and location of sampled populations (A). Bayesian tree of obtained control regions haplotypes (B). Sequences AY569543, AY569544, AY569546 were retrieved from GenBank).

PCR System 9700 (Applied Biosystems). Finally, the amplified products were purified and sequenced in the High-Throughput Genomics Unit, University of Washington.

2.3. Data analyses

2.3.1. Genetic diversity and population structure

Sequence trace files were edited in DNAStar 7.1 (DNAStar Inc., Madison, WI) and aligned by software Mega version 5.1 (Tamura et al., 2011). Sequences from the HVRI of the mtDNA control region were used to calculate genetic diversity parameters including Watterson's theta, haplotype and nucleotide diversity for each population, using DnaSP v5.10 software (Librado and Rozas, 2009). A neutrality test (Tajima's D) was performed using the same software. Additionally 33 previously published Somali wild ass sequences from Eritrea were downloaded from GenBank (Supplementary material – Table S1). Geographical structuring of the control region haplotypes was assessed by building a network, for each assumed species, using software NETWORK v4.6 (Bandelt et al., 1999).

To evaluate significant geographic divisions of hypothesized a priori species/subspecies, we have used hierarchical analyses of molecular variance (AMOVA, Excoffier et al., 1992) in ARLEQUIN v3.5 (Excoffier and Lischer, 2010). This analysis divides total variance into variance components via differences among groups (φ_{CT}), among populations within groups (φ_{SC}) and within populations (φ_{ST}). We have tested different population groupings based on both "a priori" taxonomic criteria and obtained results on our phylogenetic analyses. We assumed that the best geographic subdivisions were significantly different from random distributions and expected that the optimal genetic division of species/subspecies will maximize the between-group variance (φ_{CT}) compared to the within-group component (φ_{SC}).

2.3.2. Phylogenetic analyses and molecular dating

To clarify the existence of reciprocal monophyly among wild ass species/subspecies a phylogenetic tree of the control region haplotypes was reconstructed using Bayesian analysis with MrBAYES v3.2.1 (Ronquist et al., 2012). Individuals belonging to the most divergent HVRI haplotypes were chosen for complete sequencing of the slower evolving Cyt *b* gene for subsequent phylogenetic analyses and molecular dating of radiation events in equid species. Phylogenetic relationships among newly obtained Cyt *b* haplotypes and previously published equid sequences (Supplementary material Table S1) were inferred by using the same software. jModelTest

v2.1.3 (Darriba et al., 2012; Guindon and Gascuel, 2003) was used to select the best fitting model of molecular evolution according to the Akaike information criterion, for both sequence sets. The prior best-fitting nucleotide substitution models for the two data sets were the GTR + G + I and the HKY + G + I, for the HVRI and Cyt *b* sequences, respectively. Bayesian analyses were performed equally for both sequence sets; two independent analyses starting from different random trees were performed, and four MCMC chains were run for 50 million generations with sampling every 1000 generations. Twenty-five percent of the trees were discarded as burn-in, after checking for convergence.

The time to the most recent common ancestors (TMRCAs) of the major clades obtained in the phylogenetic analysis of complete Cyt *b* haplotypes, was estimated using a Bayesian phylogenetic framework implemented in BEAST v1.7.5 (Drummond et al., 2012). We have ran three different analyses, assuming a relaxed uncorrelated lognormal molecular clock and a Yule process of speciation. We have chosen to use only internal calibration points according to the suggestion of a recent study by Vilstrup et al. (2013). In the first analyses we have used as a calibration point for the molecular clock the emergence of the *Equus* genus (normal prior distribution centered at 4.0 My; 3–5 My 95% CI) based on the paleontological records for the monodactyle *Plesippus simplicidens*, recognized by some as the earliest fossil of the genus *Equus* (MacFadden and Carranza-Castaneda, 2002). This calibration point is further supported by recent phylogenomic studies (Orlando et al., 2013; Vilstrup et al., 2013) that point toward the origin of all extant equids between 4.0 and 4.5 Mya.

For the second analyses we have used an alternative calibration point; the emergence of the Plains zebra lineage (normal prior distribution centered at 0.7 My; 0.6–0.8 My 95% CI), according to the fossil record of *E. mauritanicus* (Eisenmann, 1979, 1980). Finally we have ran a third analyses using both previously reported calibration points and described normal prior distributions.

The nucleotide substitution model HKY + G + I (as in the phylogenetic analysis) was used in MCMC analysis. Parameters were sampled at every 1000 generations over a total of 250 million generations, with 25% generations discarded as burn-in. Convergence of the sampled parameters was checked using TRACER v1.5 (Rambaut and Drummond, 2007).

Monophyly was constrained for species represented in the analyses by more than one individual, according to obtained results of the Bayesian phylogenetic analyses.

2.3.3. Demographic dynamics

Bayesian coalescent-based methodology and MCMC sampling procedures implemented in software BEAST v1.7.5 (Drummond et al., 2012) were used to estimate the posterior distribution of population size, potentially as far back as TMCRA of a set of samples and given a determined demographic model. We chose the Bayesian skyline model (BSP) which is a piecewise-constant model of population size that allows different demographic scenarios (Drummond et al., 2005). This approach was used to make inferences about the demographic history of African and Asiatic wild asses, from mtDNA HVRI sequences, incorporating credibility intervals for the estimated effective population size at every point in time, which accounts for both phylogenetic and coalescent uncertainty. A normal distribution for the strict molecular clock prior was set with 95% of the probability density between 2.6×10^{-8} and 4.6×10^{-8} (mean 3.6×10^{-8} per generation) based on a study using the mtDNA control region of domestic donkeys and African wild asses (Kimura et al., 2011). Other parameters within the BSP model were given uniform distributions, allowing wide range variation. African wild ass samples were divided into Ethiopian and Eritrean populations and ran separately for 10^7 iterations with trees sampled every 1000 iterations. To assess the robustness of the parameter estimates, three independent chains were run with identical settings and combined into a composite chain with 2.7×10^7 states by using software LOGCOMBINER v1.4.7 (Drummond and Rambaut, 2007). The same approach was used for Iranian and Chinese wild ass populations and ran the same settings as mentioned above. In both analyses, 10% of the iterations were discarded as burn-in throughout. Log-files were analyzed in TRACER v1.5 (Rambaut and Drummond, 2007) and effective sample sizes (ESS) were used to evaluate MCMC convergence within chains.

3. Results

3.1. Genetic diversity and population structure

Analyses of 410 bp of the mtDNA HVRI region revealed 55 segregating sites among the 207 samples of African and Asiatic wild ass species, defining a total of 19 haplotypes. Obtained values for both nucleotide (π; Nei, 1987) and haplotype (h) diversities were higher in the Asiatic wild ass population of Kalimaili (China), that represents the most widespread population of *E. hemionus*, ranging from south-eastern Mongolia to north-western China. Both populations of *E. h. onager* in Iran revealed overall lower values of nucleotide diversity (0.0021 ± 0.0006 and 0.0035 ± 0.0030 for the Touran and Bahram Gour populations, respectively). Tibetan populations of *E. kiang* presented variable values of nucleotide diversity (from 0 to 0.0052 ± 0.0013 for Tingri and Nyalam populations, respectively). African wild ass populations in Ethiopia and Eritrea present vastly different diversity patterns, with nucleotide and haplotype diversity presenting considerably higher values in the Eritrean population ($\pi = 0.0082 \pm 0.0010$ and $h = 0.711 \pm 0.045$), when compared to both Ethiopian populations. Obtained values of Watterson's theta (θ_W) were overall in line with nucleotide and haplotype diversity, with the exception of the Bahram Gour population, which showed high values for this parameter and moderate low values of haplotype and nucleotide diversities. Obtained results for Tajima's D revealed a negative and highly significant value (Tajima's $D = -2.48881$; $P < 0.001$), suggesting that the Bahram Gour population might have recently begun to expand.

Geographic structuring of haplotypes revealed marked differences among Asiatic and African wild ass species (Fig. 2II). Analyses of the *E. hemionus* haplotype network showed no haplotype sharing between populations in Iran and China, however there was no clear differentiation among haplotypes belonging to both subspecies (Fig. 2IIB), with one *E. h. onager* haplotype (EH2) clustering among *E. h. hemionus* haplotypes, what might be a consequence of a recent and ongoing process of differentiation between *E. h. hemionus* and *E. h. onager* subspecies. *E. kiang* haplotype network revealed a marked east/west geographical structuring (Fig. 2IIC), with the most extreme populations of Tingri and Ngamring being represented by the two most divergent haplotypes and the southern population of Nyalam revealing two new unshared haplotypes. Clear differentiation among available *E. a. africanus* and obtained *E. a. somaliensis* haplotypes was found, however three out of four haplotypes observed in the *E. a. somaliensis* were shared between populations in Eritrea and Ethiopia revealing no geographical structure for this subspecies.

AMOVA results, showed higher among group variation in two out the four tested subdivisions scenarios (Table 2). Scenario C reflects currently accepted specific taxonomy, grouping Iranian and Chinese populations of *E. hemionus*; however this scenario performed poorly when compared to the alternative scenario D, grouping Chinese populations belonging to both *E. kiang* and *E. hemionus* species (Table 2). Scenario B tested uniquely the geographic criterion, with four clearly defined groups, corresponding to *E. africanus*, *E. h. onager*, *E. h. hemionus* and *E. kiang* populations. This scenario had the best performance explaining 85.1% of

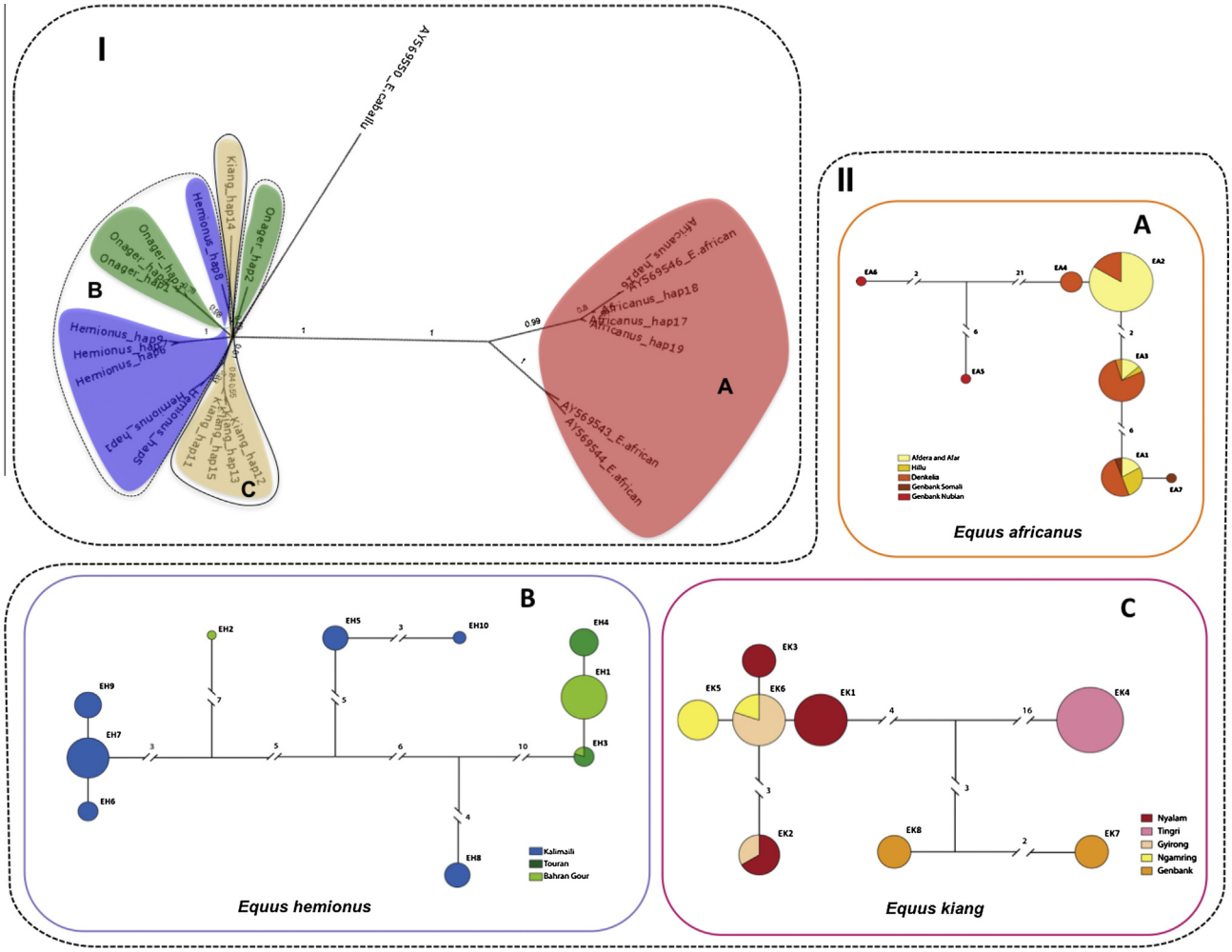

Fig. 2. Phylogenetic relationships between ass-like Equid species. I: Rooted phylogenetic network of wild ass species. Numbers above lines are Bayesian posterior probabilities. II: Median-joining mtDNA HVRI haplotype networks for each wild ass species – *Equus africanus* (A), *Equus hemionus* (B) and *Equus kiang* (C).

Table 2
Analysis of molecular variance for four subdivision scenarios according to the accepted taxonomic criteria and obtained phylogenetic results.

Scenario	Hypothesized groups	Among groups (%)	Among populations within groups (%)	Within populations (%)	$P\varphi_{CT}$
A	[Afdera, Hillu, Denkelia] [Kalamaili, Touran, Bahram Gour, Ngamring, Nyalam, Gyirong, Tingri]	78.13	16.06	5.82	0.012
B	[Afdera, Hillu, Denkelia] [Kalamaili] [Touran, Bahram Gour] [Ngamring, Nyalam, Gyirong, Tingri]	85.13	7.36	7.51	0.002
C	[Afdera, Hillu, Denkelia] [Kalamaili, Touran, Bahram Gour] [Ngamring, Nyalam, Gyirong, Tingri]	75.95	17.24	6.80	<0.001
D	[Afdera, Hillu, Denkelia] [Touran, Bahram Gour] [Kalamaili, Ngamring, Nyalam, Gyirong, Tingri]	83.10	9.92	6.98	0.001

between-group variation and simultaneously presenting the lowest within group variation (7.4%).

3.2. Phylogenetic analyses and molecular dating

Bayesian analyses of obtained wild ass HVRI haplotypes revealed two differently supported clades. The clade grouping African wild ass haplotypes, showed high posterior probability values (Fig. 1B), and haplotypes belonging to the two African wild ass lineages clustered in different branches with high support values, validating the hypotheses of two well delimited subspecies – *E. a. africanus* and *E. a. somaliensis* – in opposition to the hypothesis of one genetically uniform species. Asiatic wild ass haplotypes were grouped in a low support clade and reciprocal monophyly among proposed species/subspecies – *E. h. hemionus*, *E. h. onager* and *E. kiang* – was not recovered from the analyses. In fact, although support values for the obtained branches among Asiatic wild asses were overall low and could not fully resolve relationships among these species/subspecies, two major clades can be identified; one grouping *E. h. onager* haplotypes and the other grouping together *E. kiang* and *E. h. hemionus*. These results are not in line with other phylogenetic studies (Oakenfull et al., 2000; Steiner et al., 2012; Vilstrup et al., 2013) in which *hemionus* subspecies clustered together and *E. kiang* haplotypes formed a monophyletic clade inside the wider variation of *E. hemionus*.

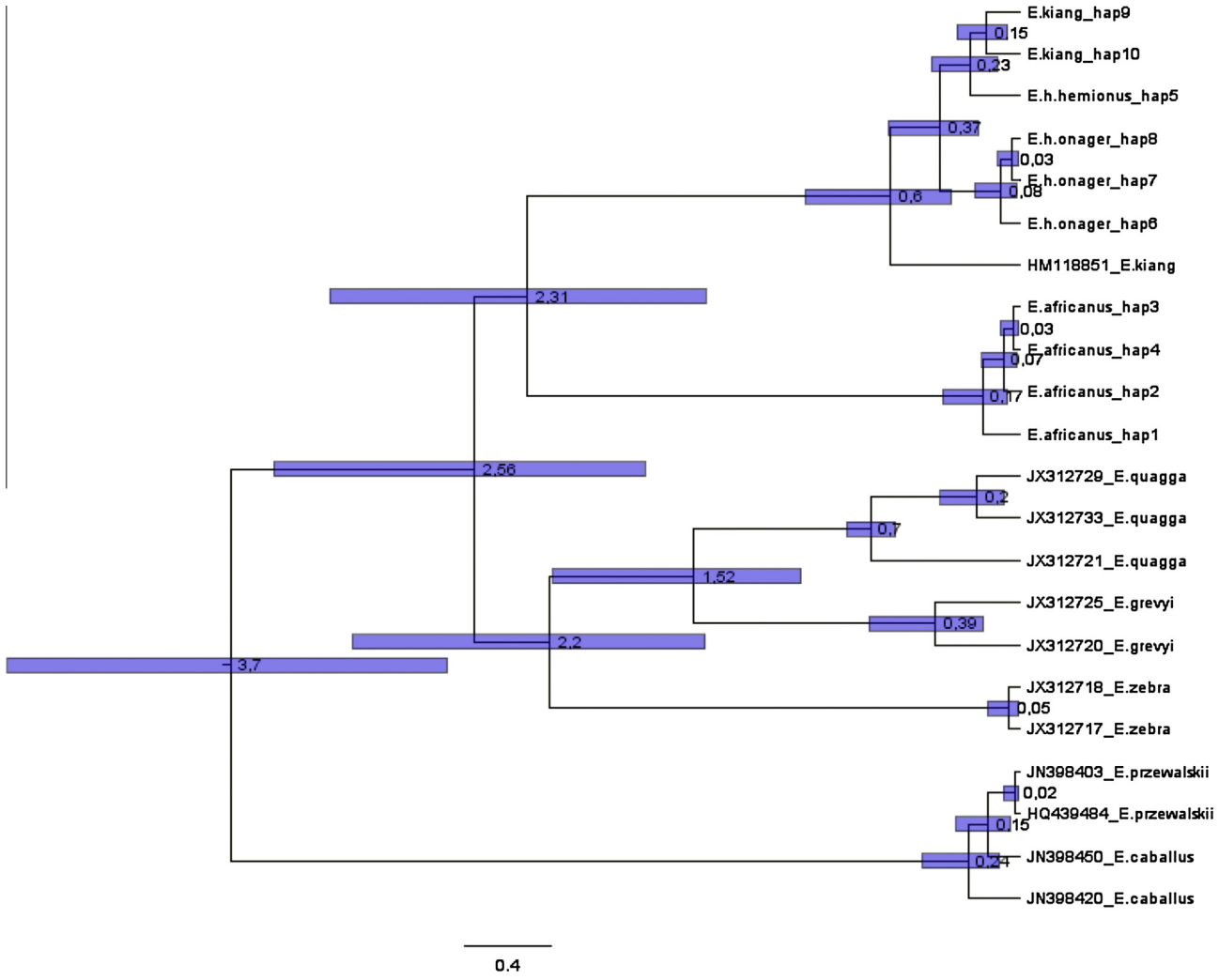

Fig. 3. Node ages, in million years, estimated by BEAST analyses of complete cytochrome *b* sequences, using two internal calibration points from the *Equus* fossil record.

Similar results were obtained in Bayesian analyses of Cyt *b* sequences, with two well-supported clades separating African and Asiatic wild ass haplotypes (Fig. 3). In the Asiatic wild ass clade it was also possible to retrieve two well supported branches that separated *E. h. onager* from *E. kiang* and *E. h. hemionus* haplotypes. Bayesian phylogenetic analyses of HVRI and Cyt *b* haplotypes are overall concordant and corroborate the hypotheses of *E. h. onager* as the most differentiated population among studied Asiatic wild ass populations.

TMCRA of the major clades obtained by phylogenetic Bayesian analyses of Cyt *b* sequences, using different calibration points within the Equidae phylogeny, were overall concordant (Table 3). Analyses incorporating the *Equus* emergence as a calibration point, revealed a shift in the time range for the arising of the genus, toward the lower limit of the prior with a geometric mean of 3.71 Mya (2.69–4.75; 95% CI). In the analyses incorporating only the calibration point corresponding to the emergence of the Plains zebra lineage, this shift was more accentuated with TMCRA of all extant equids being set at 3.18 Mya (1.4–5.0; 95% CI).

Obtained mean value for TMCRA of the wild-ass branch of the phylogeny varied from 2.08 Mya (0.99–3.27; 95% CI) to 2.37 Mya (1.26–3.55; 95% CI), depending on the calibration point used for molecular dating analyses (Table 3). Both TMCRA for the African and the Asiatic wild ass branches of the *Equus* phylogeny showed little variation, according to the calibration point used (Table 3); TMCRA of the African wild ass at approximately 200,000 years ago, is considerably younger than that for the Asiatic wild ass branch at about 630,000 years ago. These results support climatic events occurring during the Pleistocene as the major driving force in the differentiation processes in both groups of animals.

3.3. Demographic dynamics

Demographic dynamics of Asiatic and African wild ass populations revealed two different scenarios. Focal populations of Asiatic wild ass in Iran and China showed comparable demographic histories, with the parameter value $N*g$, which stands as a proxy for maternal effective population size, maintaining a relative stability until 25,000 years ago, when a population decline was detected (Figs. S2-C and S2-D). In contrast African wild ass population in Ethiopia and Eritrea remained stable over time, revealing lower effective population sizes (Figs. S2-A and S2-B), that corroborates the hypotheses of historic low population sizes and the limited distribution of this species, when compared to the closely related Asiatic wild ass.

4. Discussion

All species belonging to the *Equus* genus have a common and recent origin, with mtDNA studies identifying two deep clades, namely, the caballines and the zebras/asses (MacFadden, 2005).

Table 3
Time for the most common recent ancestor (TMCRA) of main branches obtained by Bayesian phylogenetic inference of cytochrome b sequences (Cyt *b*). All dates are in million years with 95% confidence interval given in brackets.

	Calibration point 1	Calibration point 2	Calibration point 1 + 2
TMCRA *Equus* (all species)	3.69 (2.42–4.92; 95% CI)	3.18 (1.4–5.0; 95% CI)	3.71 (2.69–4.75; 95% CI)
TMCRA Zebras (*E. quagga* + *E. zebra* + *E. grevyi*)	2.29 (1.21–3.46; 95% CI)	2.02 (1.08–3.08; 95% CI)	2.25 (1.45–3.1; 95% CI)
TMCRA Asses (*E. africanus* + *E. hemionus* + *E. kiang*)	2.37 (1.26–3.55; 95% CI)	2.08 (0.99–3.27; 95% CI)	2.34 (1.47–3.24; 95% CI)
TMCRA Horses (*E. caballus* + *E. prezwalskii*)	0.27 (0.08–0.5; 95% CI)	0.24 (0.07–0.46; 95% CI)	0.26 (0.1–0.46; 95% CI)
TMCRA African wild ass (*E. africanus*)	0.2 (0.04–0.4; 95% CI)	0.18 (0.04–0.36;95% CI)	0.19 (0.05–0.36; 95% CI)
TMCRA Asiatic wild ass (*E. hemionus* + *E. kiang*)	0.65 (0.27–1.1; 95% CI)	0.58 (0.25–0.98; 95% CI)	0.63 (0.32–1.0; 95% CI)
TMCRA Plain zebras (*E. quagga*)	0.73 (0.32–1.22; 95% CI)	0.68 (0.56–0.8; 95% CI)	0.7 (0.58–0.81; 95% CI)

These deep clades have split approximately 3 million years ago (Mya) in North America and subsequently dispersed into the Old World (MacFadden, 2005), with newly obtained genomic data suggesting that the *Equus* lineage giving rise to all contemporary horses, zebras and donkeys originated 4.0–4.5 Mya (Orlando et al., 2013; Vilstrup et al., 2013). The rich record of Pliocene equids of North America provided no support for more than one species by the early Blancan of North America, however by late Blancan (2.5–3.0 Mya) the first signs of differentiation start to appear. Scarce paleontological data from faunal assemblies refer to the possibility of a slender-limbed species existing in North America at that period of time, possibly representing the stem group of wild asses (Azzaroli and Napoleone, 1982).

In the present study we have used newly obtained Cyt *b* sequences from extant African and Asiatic species, in order to calculate TMCRA of the wild ass branch. Obtained results showed that African and Asiatic wild asses shared a common ancestor approximately 2.3 Mya (1.26–3.55; 95% CI), supporting the hypotheses of wild asses co-existing with early horses in North America, prior to dispersal to the Old World. These results seem to be in good agreement with a recent study that analyzed mitogenomes from all extant equid lineages which places the common ancestor of the wild-ass branch at 2.6 Mya (Vilstrup et al., 2013).

Equus is believed to have dispersed to Eurasia, before the end of the Pliocene, arriving to India by 2.5 Mya, Western Europe simultaneously or even slightly earlier and East Africa at about 2 Mya (Azzaroli, 1992; Lindsay et al., 1980). If this was the case, then the origin of wild asses and the first *Equus* dispersal events to the Old world happened approximately at the same time range, raising the hypothesis that competition for habitat and resources triggered the first long range dispersal events.

Our Bayesian phylogenetic analyses of Cyt *b* sequences resulted on a well-supported unrooted tree (Fig. S1), in which the African wild branch appears as a sister lineage to Asiatic wild asses. Both African and Asiatic wild ass branches were found monophyletic with high posterior probability values. This pattern is consistent with other studies (McCue et al., 2012; Steiner et al., 2012; Vilstrup et al., 2013) that also support African and Asiatic wild asses as sister species. Among Asiatic wild asses, we were unable to retrieve *E. kiang* monophyly, and instead found *E. h. onager* haplotypes to cluster inside the wider *E. hemionus* variation in a well-supported monophyletic clade. These results are in clear disagreement with recent studies (Steiner et al., 2012; Vilstrup et al., 2013) that show *E. hemionus* subspecies clustering together in a group divergent from *E. kiang*. Such inconsistencies might be a result of incomplete lineage sorting among Asiatic wild asses, resulting in random phylogenetic reconstructions. Despite incongruences in tree topology, TMCRA of the Asiatic wild branch 630 Kya (thousand years ago) is in clear agreement with that obtained byVilstrup et al. (2013) at 672 Kya. TMCRA of the African wild ass at 200 Kya was much younger than previously published results (Oakenfull et al., 2000; Vilstrup et al., 2013), however we have used sequences belonging only to the Somali wild ass lineage (*E. a. somaliensis*) what could account for this discrepancy.

African and Asiatic wild ass species have undergone different evolutionary processes. The African wild ass seems to be less successful when compared with the closely related Asiatic ass, showing a historically more restricted distribution, in marginal arid habitats in the Horn of Africa. On the other hand, Asiatic wild asses were widely distributed from the Asiatic Far East (southeastern Mongolia) into the Near Eastern Mediterranean shores and as far south as central Arabian Peninsula and the North of the Indian Subcontinent, thus occupying a vast territory.

Asiatic wild ass populations of *E. hemionus* have been stable until approximately 25,000 years ago, when a population decline is observed (Figs. S2-C and S2-D). This decline coincides with the time of the last maximum glacial (LGM), between 19,000 and 26,000 years ago, when large mammals vanished from many biogeographic regions, finding refuge or shifting distributions to the southern extreme of their range. Although it has been argued that East Asia has never been covered in ice sheets during the last glaciation events (Zhang et al., 2008) mainly due to the monsoon effect, the indirect impact of global climatic change most probably affected all parts of the world (Lister and Stuart, 2008). After climatic conditions improved, *E. hemionus* populations would have the capacity to recover, however by then the human population had already occupied many of the areas inhabited by these animals and hunting and habitat loss continued to impose a decreasing trend until current days. Similar results were found for closely related wild horses (Lorenzen et al., 2011) that showed a decline in genetic diversity after the LGM, reflecting the impact of expanding human populations in Europe and Asia.

Comparatively, the African wild ass apparently went through different demographic dynamics. Perhaps due to their more restricted distribution in a region of the globe less affected by the climatic events, African wild ass populations have managed to keep stable effective population sizes over time.

The current levels of genetic diversity among wild ass populations reflect a very intricate evolutionary process. For instances, the Iranian populations of *E. h. onager* presented low genetic diversity as a consequence of the accentuate decrease in numbers during the course of the last decades, essentially due to habitat destruction, overgrazing and poaching (Moehlman et al., 2008a, 2008b). Iranian populations are now separated by more than 700 km, subsisting in isolation what is an added concern for their survival. Diversity levels are considerably higher in the Kalimaili population of *E. h. hemionus*, which represents the most widely distributed subspecies of Asiatic wild asses. Despite the apparent genetic health, *E. h. hemionus* populations have been losing suitable habitat across their current range and populations in Mongolia and China are declining and becoming increasingly isolated. Diversity levels in populations of *E. kiang* in Tibet are highly variable (Table 1), however their distribution across the isolated region of the Tibetan plateau, makes them less vulnerable to human mediated actions. Obtained geographical structure of control region haplotypes in *E. kiang* populations, in a comparably smaller area than the wider Iran-China distribution range of the *hemionus spp*. might reflect the unique features of the habitat occupied by these populations,

with altitude and harsh mountain slopes working as geographic barriers and promoting differentiation.

E. kiang and *E. hemionus* have allopatric distribution ranges and share morphological, ecological and behavioral similarities, however on the bases of coat color and karyotypic dissimilarities, they have been considered different species. Our phylogenetic analyses of Cyt *b* haplotypes revealed a clear pattern of differentiation between *E. h. onager* and the cluster that incorporates *E. kiang* and *E. h. hemionus* haplotypes (Figs. 3 and S1). AMOVA results also supported these results, with the geographical criterion overpowering the taxonomic groupings of different Asiatic wild ass species/subspecies. The larger percentage of variation justified by differences between groups is obtained when *E. kiang*, *E. h. hemionus* and *E. h. onager* are placed in different groups (scenario B – Table 2) and when *E. kiang* and geographically closest population of *E. h. hemionus* are grouped together (scenario D – Table 2).

E. africanus is currently the most threatened equid species, with less than 500 individuals subsisting in Ethiopia and Eritrea (Moehlman et al., 2008a, 2008b). Nubian wild asses (*E. a. africanus*) are extremely rare and may already be extinct, however exploratory trips in the northern regions of Eritrea need to be done. In fact, the lack of extensive sampling in contemporary populations has been an obstacle to fully understand the level of differentiation among African wild asses. By sampling Eritrean and Ethiopian populations of *E. a. somaliensis*, we were able to clearly demonstrate the absence of geographical structure, with populations across the border sharing three of the four obtained HVRI haplotypes. Gene flow among *E. a. somaliensis* populations is possible given the lack of geographical barriers. The lack of geographic structure in HVRI haplotypes indicates that these populations were most probably connected and gene flow may still occur. Comparison of obtained haplotypes with previously published *E. a. africanus* haplotypes, revealed clear differentiation, reinforcing the hypotheses of two well defined subspecies, corresponding to the Nubian and Somali lineages.

5. Conclusions

Here, we have shown that by using an integrative approach that combines non-invasive sampling together with phylogenetic and phylogeographic methodologies it is possible to obtain new and more complete insights into the evolutionary history of wild ass species. Besides the important contribution to knowledge on historical processes that led to current genetic variation, this work could serve as a framework for the development of conservation actions in wild ass populations and furthermore call for a reevaluation of taxonomic status in Asiatic wild ass species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.01.005.

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